Supporting Information

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SI Materials and Methods

Xenopus Extracts and DNA Immunoprecipitation. For extracts treated with DSB DNA, 5 μL of 0.01 mM dAdT dsDNA (70 nt) was added to 50-μL extracts. Extracts were incubated at room temperature. For DNA precipitations, 1 μL of biotin-tagged dsDNA (0.25 mM) was bound to 25 μL of avidin beads for each reaction. DNA-bound beads then were incubated with 100-μL extracts for 20 min at room temperature. Beads were removed from extracts and washed four times with egg lysis buffer (250 mM sucrose, 2.5 mM MgCl, 1 mM DTT, 50 mM KCl, and 10 mM Hepes, pH 7.7).

Cell Culture and Drug Treatments. 293T, HeLa, or MCF7 cells were maintained in high-glucose DMEM medium supplemented with 10% FBS. PC3 cells were maintained in F12K medium. Cells were grown to 90% confluence before treatment with drugs. For SL0101 treatment, 1 μL of 100 mM SL0101 was added to 1 mL of medium for a final concentration of 100 uM. Cells were treated with SL0101 for 30 min. For phorbol12-myristate13-acetate (PMA) treatment, 0.5 μL of PMA (50 ng/μL) was added to 1 mL of medium to make final a concentration of 25 ng/mL Cells were treated for 30 min. For neocarzinostatin (NCS) treatment, 0.2 μL of NCS (500 ng/mL) was added to 1 mL medium to make a final concentration of 200 ng/mL After treatment for 30 min, cells were washed once with PBS.

Transfection and siRNA. FuGENE 6 (Roche) was used to transfect cells with plasmids according to the manufacturer's instructions. Lipofectamine RNAiMAX (Invitrogen) was used to perform siRNA transfections. siRNA targeted against ribosomal s6 kinase 1 (RSK1) and RSK2 as previously described (40) were purchased from Sigma.

Plasmid Reagents and Antibodies. C-terminal HA-tagged meiotic recombination 11 (Mre11) and N-terminal Flag-tagged RSK2 were cloned into pCDNA3 plasmid. Mre11 alanine mutants and the constitutively active RSK2 707A mutant were generated by site-directed mutagenesis. The antibodies against human Mre11 (Abcam), phospho-S6 Ser235/236 (Cell Signaling), phosphor-histone H3 (Ser10) (Cell Signaling), actin (Santa Cruz), phosphor-Mre11 Ser-676 (Cell Signaling), goat anti-HA (Abcam), His-probe (Santa Cruz), γH2AX Ser139 (Cell Signaling), human ataxia telangiectasia mutant (ATM) (Abcam), phospho-ATM Ser1981 (Epitomics), and anti-human/*Xenopus* Mre11 (Calbiochem) were obtained as stated.

The antibodies against *Xenopus* Nijmegen breakage syndrome 1 (Nbs1) and ATM were gifts from William Dunphy (California Institute of Technology, Pasadena, CA).

The following drugs were used: PMA (Enzo Life Science), NCS (Sigma), SL0101 (Calbiochem), and Ku55933 (Calbiochem).

Biotin-tagged ssDNA was purchased from Integrated DNA Technologies. Biotin-tagged dsDNA was synthesized by annealing tagged ssDNA with its anti-sense DNA at 70 °C and gradual cooling to room temperature.

Kinase Assay. For kinase assays in mammalian cell lysates, nickel-agarose–bound Mre11-His was incubated with 2 μCi γ-[^{32}P]ATP in cytosolic lysates from mammalian cells transfected with RSK2 for 1 h at 30 °C. Beads then were removed and washed three times in washing buffer [1 mM Hepes-KOH (pH 7.5), 1 mM β-glycerophosphate, 15 mM KCl, 0.1% Nonidet P-40, 14.8 mM imidazole, 500 mM NaCl, 1 mM $NaVO_3$, and 5 μg/mL aprotinin/leupeptin].

For the in vitro kinase assay, nickel-agarose–bound Mre11-His protein was incubated in kinase buffer [10 mM Tris·HCl (pH 7.5), 0.1 mM ATP, 10 mM $MgCl_2$, 1 mM DTT (pH 7.2)] with 2 μCiγ-[^{32}P]ATP and RSK2 kinase (Millipore). After 30 min at 30 °C, the beads were washed with washing buffer. Analysis for all kinase assays was performed using SDS/PAGE and autoradiography.

Immunoblot Analysis. For tissue culture, cells were cultured to 90% confluence before harvesting with trypsin. Cells then were lysed with extraction buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 12.5 mM M glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 20 μM aprotinin, 1 mM PMSF, and 0.5% Triton X-100]. The concentration of cell lysates was measured. Equal amounts of lysates were loaded for SDS/PAGE. Proteins were transferred from gels to PVDF membrane and were immunoblotted with antibodies. For large-molecular-weight proteins such as ATM, low-voltage (50 V) was used in the overnight transfer.

For *Xenopus* extracts, 5 μL of extract was mixed with 50 μL SDS sample buffer. Extracts and samples were loaded for SDS/PAGE, transferred from gels to PVDF membrane, and processed for immunoblotting with antibodies.

FACS. 293T cells were treated with inhibitors as describe in *Results* in the main text and after 22 h were fixed with 4% paraformaldehyde for 20 min. Cells then were permeabilized with 0.2% Triton in PBS buffer. After washing, cells were incubated with primary antibodies at 37 °C for 2 h and then with secondary antibody for 45 min. Cells were washed with PBS, treated with RNase for 15 min, and stained with propidium iodide (PI). Cell profiles were analyzed by flow cytometry. Each experiment was repeated three times, and the Student *t* test was applied to assess statistical significance. Significance levels are indicated in *Results* and figure legends.

Additional Information for Microscopy. For microscopy, a Zeiss Axio Imager widefield fluorescence microscope equipped with a mercury arc lamp (Zeiss HBO100 power supply and lamp housing) was used. The filter cube sets were Chroma 31000v2 (excitation wavelength range: 325 nm–375 nm, beam splitter: 410 nm, emission wavelength range: 435 nm–485 nm) for DAPI; Chroma 41017 (excitation wavelength range: 450 nm–490 nm, beam splitter Q495LP, emission wavelength range: 500 nm–550 nm) for GFP; and Chroma 41004 (excitation wavelength range: 535 nm–585 nm, beam splitter 595 nm: emission wavelength range, 610 nm–680 nm) for RFP. Objectives used were a Zeiss 20×/0.50 EC Plan-NeoFluar and a Zeiss 40×/0.75 EC Plan-NeoFluar. The camera was a Hamamatsu Orca ER monochrome cooled-CCD camera. The acquisition software was MetaMorph Premier version 7.7.3.0. Fluorescent intensity in the original raw images was quantified with MetaMorph. Blank areas within images were selected as background during quantification. Images also were adjusted for contrast and pseudocolored in MetaMorph for presentation in figures.

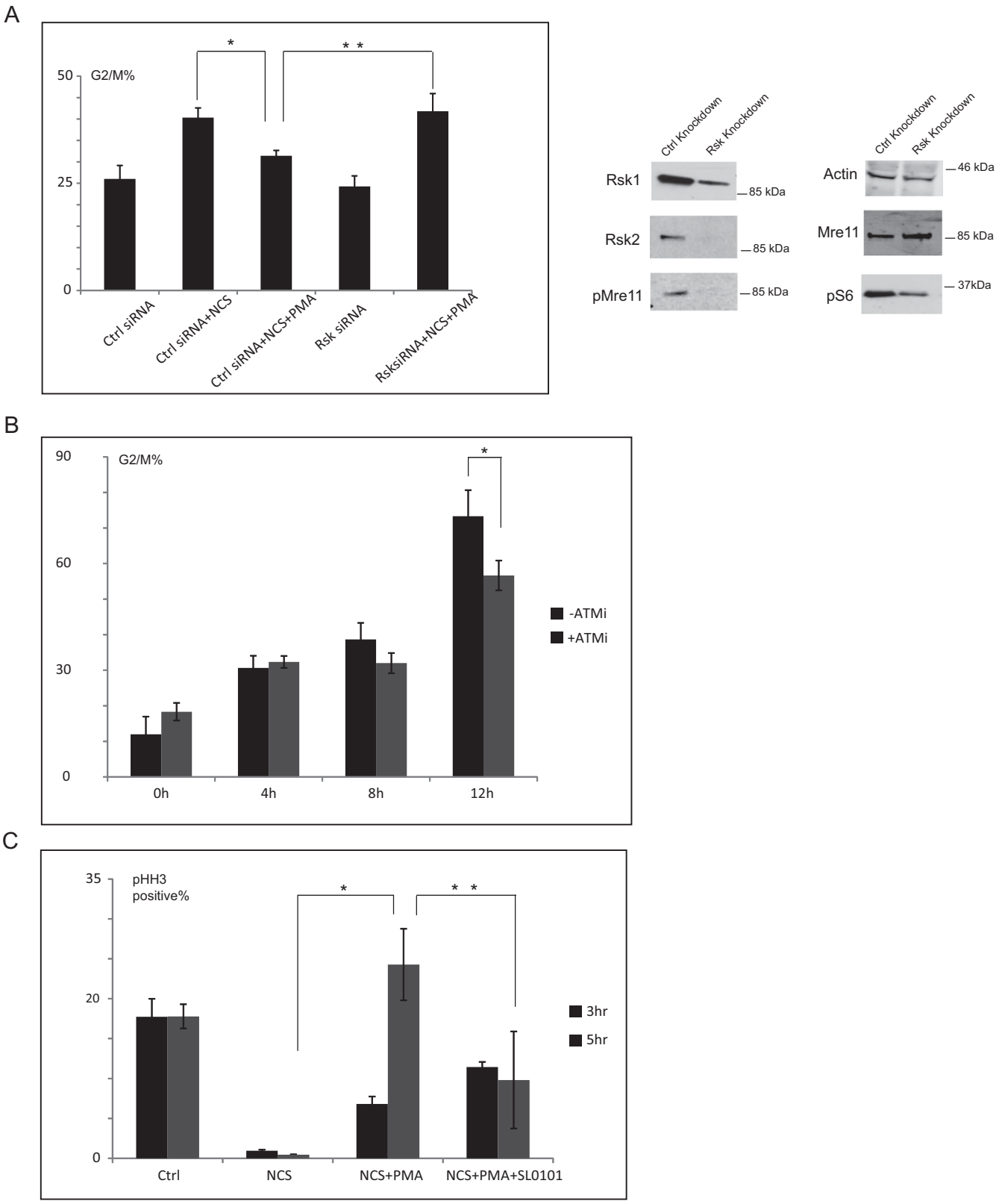

Fig. S1. (*A*) 293T cells were transfected with RSK1/2 siRNA. Forty-eight hours later, cells were treated with PMA for 30 min and then with NCS for another 30 min. (*Left*) Cells were collected after 16 h, and DNA profiles were analyzed by flow cytometry. (*Right*) The effectiveness of knockdown was analyzed by Western blotting. Data from three experiments were combined. $*P < 0.01$; $**P < 0.01$. Error bars indicate SD. (*B*) HeLa cells were treated with ATM inhibitor for 30 min and then with NCS for another 30 min. Cells were collected at the indicated time points, and DNA profiles were analyzed by flow cytometry. Data from three experiments were combined. $*P < 0.01$. Error bars indicate SD. (*C*) HeLa cells were synchronized in G1/S phase by double thymidine block. Four hours after release, cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min. Three or five hours later cells were collected and stained

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with anti–phospho-Histone H3 antibody (pHH3). The pHH3-positive cells were analyzed by flow cytometry. Data from three experiments were combined. *$P <$ 0.001; **$P < 0.01$. Error bars indicate SD.

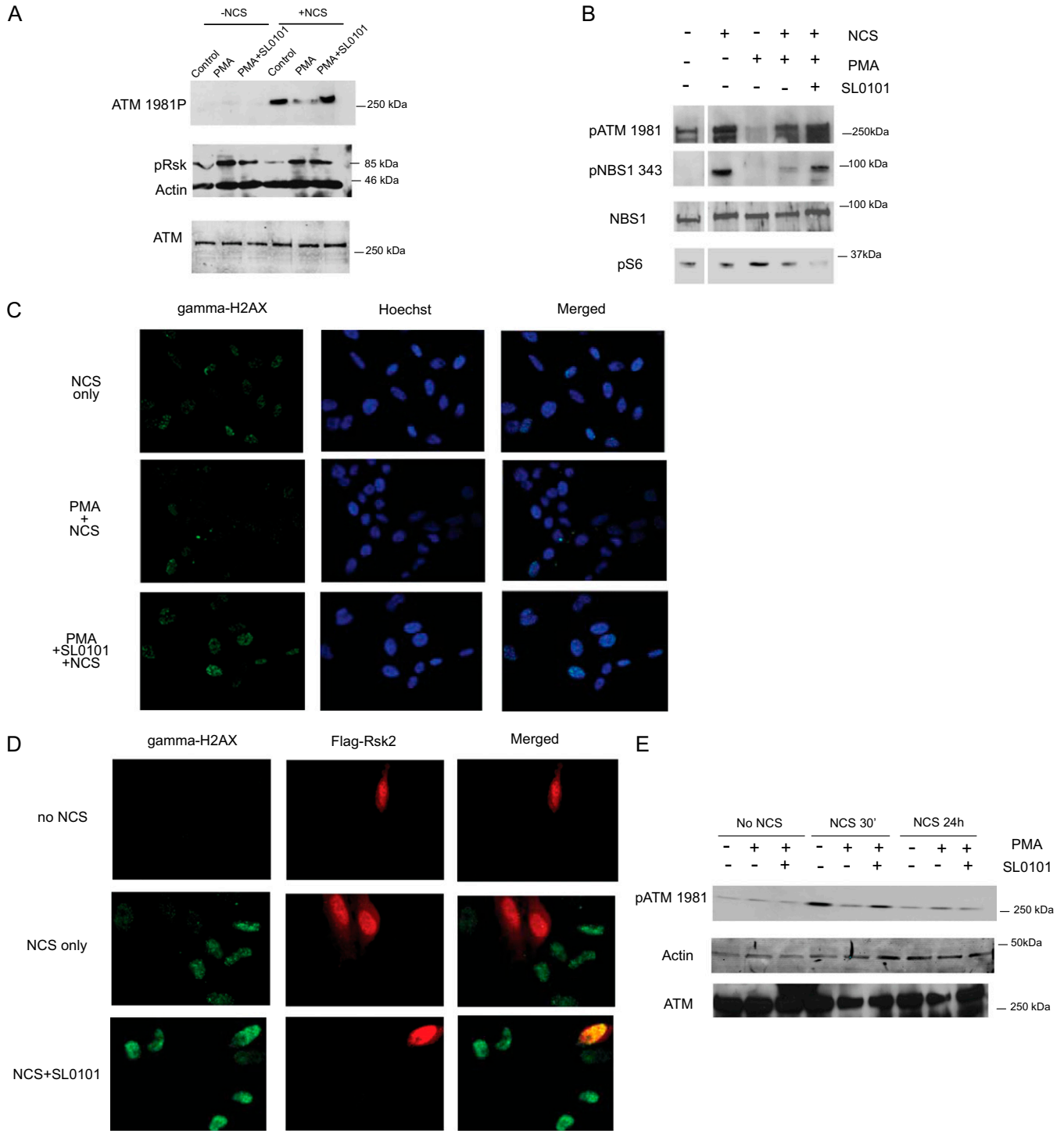

Fig. S2. (*A*) 293T cells were treated with PMA and SL0101 or with PMA alone for 30 min and then with NCS for another 30 min before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981. (*B*) HeLa cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min before harvesting. Cell lysates were immunoblotted for phospho-NBS1 Ser-343 and for phospho-ATM. (*C*) HeLa cells were stained with DAPI and rabbit anti-γH2AX. The pattern of γH2AX staining was examined by fluorescence microscopy. Images were taken under a 20× objective. (*D*) HeLa cells were transfected with FLAG-tagged constitutively active RSK2 and costained with mouse anti-FLAG and rabbit anti-γH2AX antibody. The pattern of γH2ax staining in FLAG-positive and -negative cells was examined by fluorescence microscopy. Images were taken under a 40× objective. (*E*) HeLa cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min. Cells were harvested immediately or were cultured in regular medium for another 24 h before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981.

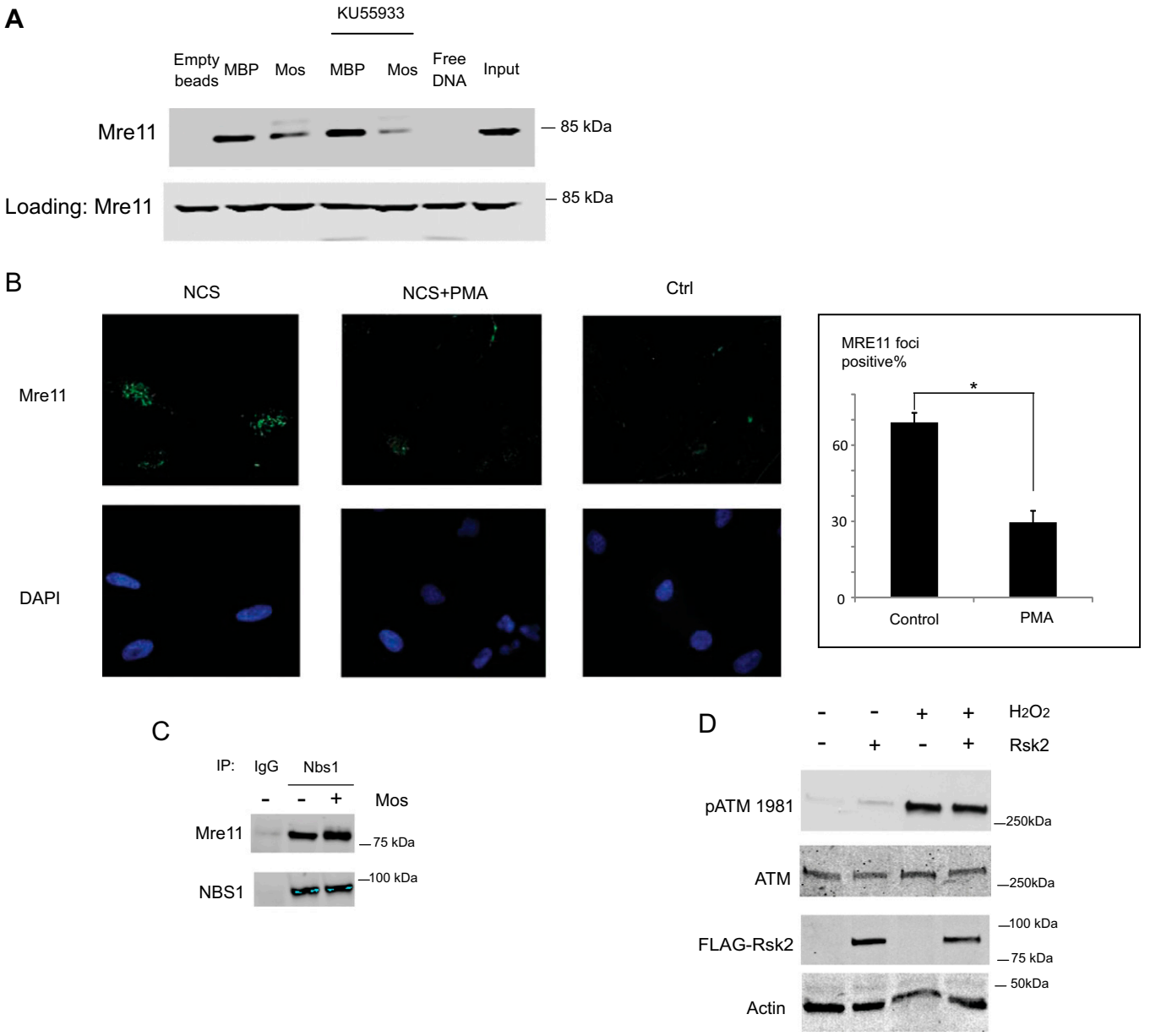

Fig. S3. (*A*) Biotin-tagged dsDNA was bound to avidin beads and added to *Xenopus* crude S extracts pretreated with maltose-binding protein (MBP)-Mos or with MBP and then KU55933 and were incubated for 20 min. In one of the reactions, free dsDNA was added to compete with the DNA on the beads. The amount of Mre11 protein bound to beads was analyzed by immunoblotting. (*B*) HeLa cells were grown on coverslips and were treated with PMA for 30 min and then with NCS for another 10 min before fixation. (*Left*) Cells were stained with DAPI and rabbit anti-Mre11. The pattern of Mre11 foci staining was examined by fluorescence microscopy. Images were taken under a 40× objective. Cells with more than five clear Mre11 foci were counted as Mre11-positive. (*Right*) Results from three experiments were combined. Fifty cells from at least 10 random fields were counted in each experiment. $*P < 0.001$. Error bas indicate SD. (*C*) Nbs1 protein was immunoprecipitated from *Xenopus* extract pretreated with Mos or MBP. Mre11 protein bound to Nbs1 was immunoblotted. (*D*) HeLa cells transfected with FLAG-tagged Rsk2 were treated with 0.9 mM H_2O_2 for 30 min before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981.

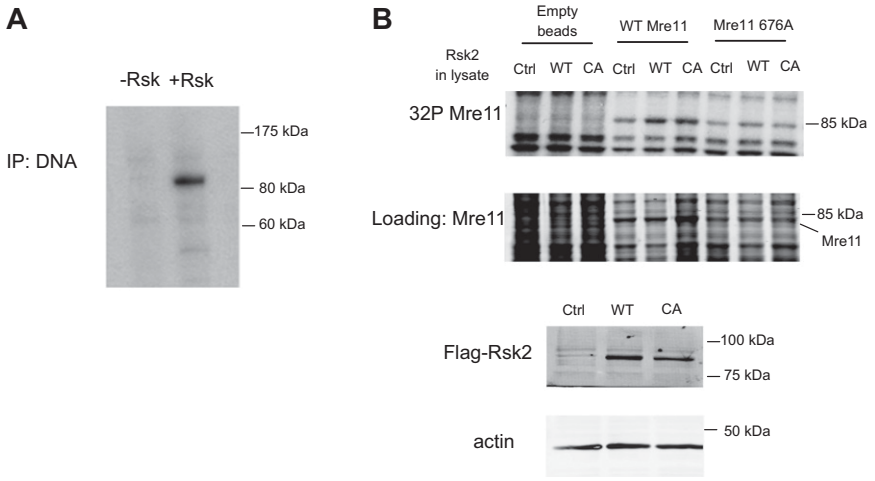

Fig. S4. (*A*) Biotin tagged-dsDNA was bound to avidin beads and incubated with *Xenopus* crude interphase egg extract for 20 min. After washing with egg lysis buffer, beads were spilt and treated with RSK2 kinase and γ-[^{32}P]ATP or with γ-[^{32}P]ATP alone as a control. Proteins were resolved in SDS sample buffer and analyzed by autoradiography. (*B*) 293T cells were transfected with WT Rsk or constitutively active Rsk (RSK CA). Cells were collected 2 d after transfection to make lysates. Equal amounts of WT His-tagged Mre11 protein and the 676A mutant proteins were mixed with lysates, kinase buffer, and γ-[^{32}P]ATP at 37 °C for 45 min. Radiolabeled Mre11 protein was analyzed by autoradiography.

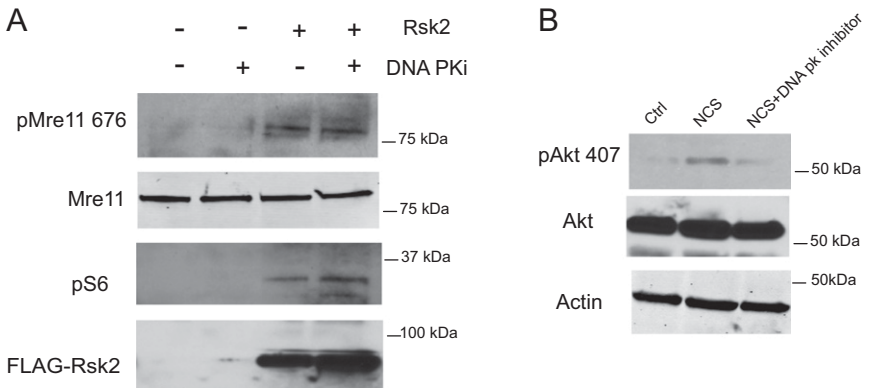

Fig. S5. (*A*) HeLa cells transfected with active Rsk2 kinase were treated with DNA PKC inhibitor for 30 min before harvesting. Lysates were blotted for anti-Mre11 Ser-676 antibody. (*B*) HeLa cells were treated with DNA PKC inhibitor for 30 min and then with NCS for another 30 min before harvesting. Lysates were immunoblotted with anti-Akt Ser407 antibody to see whether phosphorylation of Akt by DNA PKc was inhibited.

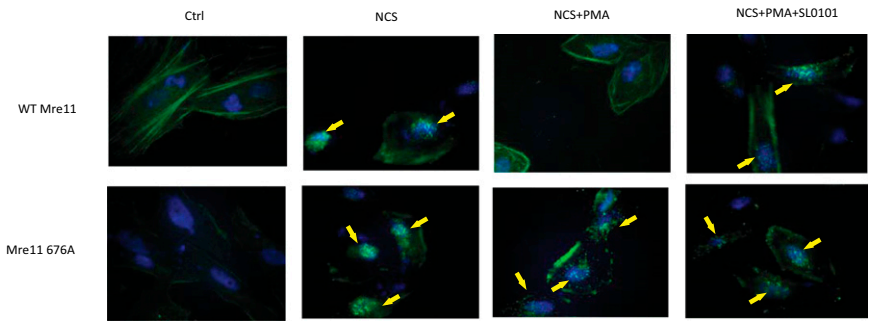

Fig. S6. HeLa cells infected with Mre11 shRNA were grown on coverslips and transfected with shRNA-resistant WT Mre11 or 676A mutant. Forty-eight hours after transfection, cells were treated with PMA and SL0101 for 30 min and then with NCS for another 10 min before fixation. Cells were stained with DAPI and rabbit anti-Mre11 antibody. The pattern of Mre11 foci staining was examined by fluorescence microscopy. Images were taken under a 40× objective. Shown are representative images of more than 50 cells from at least eight random fields from three experiments.(See Fig. 5*F* for quantitation.) Yellow arrows indicate Mre11 foci in the nuclei.

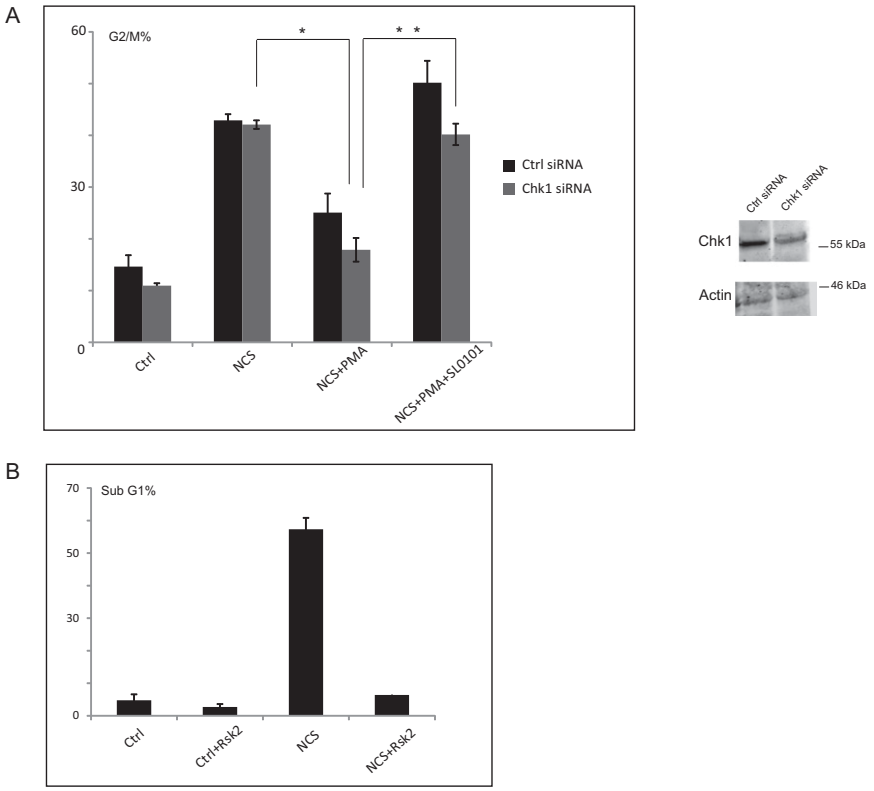

Fig. S7. (*A*) 293T cells were treated with siRNA to knock down endogenous checkpoint kinase 1 (Chk1). Forty-eight hours later, cells were treated with PMA and/or SL0101 for 30 min and then with NCS for another 30 min. Cells were grown in DMEM medium for 16 h before harvesting, and DNA profiles were analyzed by flow cytometry. Data from three experiments were combined. $*P < 0.01$. Error bars indicate SD. Note that the knockdown of Chk1 had minimal effect on the response to NCS, possibly because the ATM/Chk2 pathway, the primary pathway for the dsDNA-break response, remained intact. (*B*) HeLa cells were transfected with constitutively active Rsk2 kinase. After 36 h, cells were treated with 200 ng/mL NCS for 1 h. Cells were grown in DMEM medium for another 48 h before harvesting. DNA profiles were analyzed by flow cytometry, and the sub-G1 population was analyzed. Data from three experiments were combined. Error bars indicate SD.